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Binding of 5,5′-Bis[8-(phenylamino)-1-naphthalenesulfonate] by the Regulatory Subunits of Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase†

Janice L. Bohnert, Dean A. Malencik, Sonia R. Anderson, David Teller, and Edmond H. Fischer*

ABSTRACT: Binding to the regulatory subunits of types I and II adenosine cyclic 3′,5′-phosphate (cAMP) dependent protein kinase (R^I and R^{II}, respectively) produces large distinctive increases in fluorescence and optical activity of 5,5′-bis[8-(phenylamino)-1-naphthalenesulfonate] [bis(ANS)]. Both specific and nonspecific interactions are involved. Association of the regulatory subunits with either the catalytic subunit or cAMP results in dissociation of a major portion of the bound bis(ANS) as detected by changes in fluorescence and circular dichroism. The results are consistent with the accepted cAMP binding properties of R^I and R^{II}, showing cooperativity in the case of R^I and two heterologous binding sites for R^{II}. cGMP has the same overall effect on bis(ANS) binding as cAMP. However, very high concentrations are required for complete dissociation of bis(ANS) from R^{II}, consistent with the observation that cGMP is inefficient in bringing about the dissociation of the type II holoenzyme. Magnesium binding to sites having dissociation constants of ca. 12 mM increases the interaction of bis(ANS) with both of the isolated regulatory subunits. Experiments involving the 37 000-dalton fragment of R^{II} indicate that the limited proteolytic cleavage was heterogeneous, with only 24–39% of the resulting population interacting strongly with the catalytic subunit.

In the preceding paper (Bohnert et al., 1982), the fluorescent probe 1,N^6-ethenoadenosine cyclic 3′,5′-phosphate (ϵ-cAMP)[1] was used to study the interaction of the regulatory subunits of protein kinase with the catalytic subunits (C) and to demonstrate the effects of molecules which bind to C (MgATP, substrate, and protein kinase inhibitor) on the R–C interaction.

† From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331, and the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received April 26, 1982.* This work was supported by research grants from the Muscular Dystrophy Association and from the National Institutes of Health (AM 13912, AM 07902 and GM 13401). It was taken in part from a thesis presented to the Graduate Faculty of the University of Washington in partial fulfillment of the requirements for the degree Doctor of Philosophy.

* Correspondence should be addressed to this author at the Department of Biochemistry, University of Washington.

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; cAMP, adenosine cyclic 3′,5′-phosphate; ϵ-cAMP, 1,N^6-ethenoadenosine cyclic 3′,5′-phosphate; bis(ANS), 5,5′-bis[8-(phenylamino)-1-naphthalenesulfonate]; ANS, 8-anilino-1-naphthalenesulfonate; $NaDodSO_4$, sodium dodecyl sulfate; R^I and R^{II}, regulatory subunit of types I and II cAMP-dependent protein kinase, respectively; $38K^I$ and $37K^{II}$, carboxy-terminal fragment of the regulatory subunit of types I and II cAMP-dependent protein kinase, respectively; C, catalytic subunit of the cAMP-dependent protein kinase; CD, circular dichroism; F_∞, fluorescence of totally bound ligand; F_0, fluorescence of unbound ligand; F, observed fluorescence; ϕ, fractional degree of saturation; $\bar{n}$, number of moles of ligand bound per mole of protein; BSA, bovine serum albumin.

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This report describes the application of a different fluorescent probe, 5,5′-bis[8-(phenylamino)-1-naphthalenesulfonate] [bis(ANS)], to a comparative study of R^I and R^{II} and their proteolytic fragments. Bis(ANS) is a near-dimer of 8-anilino-1-naphthalenesulfonate (ANS) first synthesized and described by Rosen & Weber (1969). Like ANS, bis(ANS) is virtually nonfluorescent in aqueous solutions but highly fluorescent in nonpolar solvents and in specific protein adsorbates. Anderson (1971) and Lu & Anderson (1973) used bis(ANS) to study the five isozymes of lactate dehydrogenase. Although the two subunit types, H and M, exhibited characteristically different behavior in the presence of bis(ANS), the binding of the dye by all the isozymes was sensitive to NADH. Sedimentation experiments showed displacement of bis(ANS) by NADH, suggesting that the dye is useful as a probe of nucleotide binding sites. The experiments described herein demonstrate that, while the binding is heterogeneous, both types of regulatory subunits of protein kinase and their fragments bind bis(ANS) in the micromolar concentration range. As with lactate dehydrogenase, the fluorescence and circular dichroism of the bound bis(ANS) are sensitive to the interaction of the protein with other molecules. With the regulatory subunits, the measurements are influenced by the binding of cAMP, cGMP, Mg^{2+}, and the catalytic subunit. The results emphasize the similarities and differences between the two types of regulatory subunit while providing information on the relationship of the two enzymes to the cGMP-dependent protein kinase.

Materials and Methods

Materials. ANS (8-anilino-1-naphthalenesulfonate) was purchased from Eastman Kodak and the anionic resin AGl-X8 (200–400 mesh) from Bio-Rad. All other reagents are described in the preceding paper (Bohnert et al., 1982), together with the preparation of all enzyme components and other analytical procedures.

Bis(ANS) was prepared according to an extensive modification of the original procedure of Rosen & Weber (1969). Four liters of 50 mM KH_2PO_4 was adjusted to pH 2 by the addition of concentrated H_3PO_4. To this solution was added 5 g of bovine serum albumin and 150 mg of ANS. After all the reagents had dissolved, 2 mL of 50 mM $NaNO_2$ was added and the reaction allowed to proceed for 30 min. The pH was then adjusted to 7 with concentrated NaOH. The solution was concentrated on an Amicon apparatus with a YM-30 membrane. After 100-fold concentration, 2.5 volumes of 95% ethanol was added, and the temperature of the solution was raised to 65 °C for 5 min. After the solution was cooled to 4 °C, it was centrifuged for 10 min at 3000*g*. The resulting supernatant was evaporated and the residue taken up in a minimum of water. The solution was then brought to 0.25 M in KCl, and a precipitate was allowed to form overnight in the cold. The suspension was centrifuged in a table-top centrifuge and the supernatant placed on a neutral alumina column (Bio-Rad) equilibrated with 70% methanol and 30% 10 mM potassium phosphate buffer, pH 7.0.

Three main peaks were collected from the column; the first peak to emerge contained ANS and the second, bis(ANS). These were followed by a dark brown material. After the appropriate tubes containing bis(ANS) were pooled on the basis of its characteristic fluorescent properties with bovine serum albumin (Rosen & Weber, 1969), the solution was evaporated. The dried powder was taken up in a minimum of water and allowed to crystallize. After two more crystallizations, the bis(ANS) was pure as judged by thin-layer chromatography in several solvent systems. Comparison of its protein binding properties to those of a sample of bis(ANS) donated by Professor G. Weber showed it to be identical and free of ANS. Approximately 25 mg of bis(ANS) was recovered, corresponding to a yield of 30%.

Table I: Fluorescence Properties of Bis(ANS) Bound to Regulatory Subunits, Fragment, and Holoenzyme[a]

sample	λ_{max} (nm)	Q[b]
R^I	504	0.50
R^{II}	504	0.63
$37K^{II}$	510	0.63
$R^I{}_2C_2$	505	0.61
$R^{II}{}_2C_2$	491	0.95
BSA	498	0.70[c]

[a] Conditions were 5 mM Mops, 0.1 N KCl, 50 mM β-mercaptoethanol, and 1 μM bis(ANS), pH 7. [b] Quantum yields were extrapolated to infinite protein concentration. [c] From Rosen & Weber (1969).

Binding experiments were carried out in the presence of 0.1 M KCl, 15 mM β-mercaptoethanol, 5 mM Mops, pH 7.0, and the indicated concentrations of $MgCl_2$, ATP, cAMP, ϵ-cAMP, and cGMP, at 20 °C. Glass-distilled water was used throughout.

Fluorescence measurements were obtained with a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. The excitation and emission wavelengths used with bis(ANS) were generally 390 and 480 nm, respectively. A single experiment with 1.2 mg/mL R^{II} used excitation at 306 nm, an isosbestic point, in order to lower the total absorbancy. In the energy transfer experiments, tryptophan standards having the same absorbancies as the protein samples were titrated with bis(ANS) to determine the correction factors for the inner filter effect.

Sedimentation velocity experiments were performed in a Beckman-Spinco Model E analytical ultracentrifuge equipped with interference optics and scanner. Binding measurements of bis(ANS) to R^{II} were done at 360 nm. The details of the calculations and the other procedures were described earlier (Bohnert et al., 1982).

Circular dichroism spectra were recorded on a Dichrograph Mark III circular dichroism recorder and spectrophotometer under nitrogen flush at 20 °C. A cuvette with 1-cm path length was used. The values of $\epsilon_L - \epsilon_R$ were calculated by using the total bis(ANS) concentration.

Results

General Description of Bis(ANS) Binding. Table I shows that the adsorbates of bis(ANS) with the regulatory subunits, the fragment $37K^{II}$, and the holoenzymes have quantum yields in the same range as that obtained with bovine serum albumin. Figure 1 illustrates fluorescence titrations of the proteins with bis(ANS). The data are presented as plots of $(\bar{q}/q_0)(X_0/P_0)$ vs. X_0/P_0 where X_0 is the total concentration of bis(ANS) and P_0 the total protein concentration in terms of regulatory subunit monomer. Calculations of binding from fluorescence data are based on a two-state model relating fluorescence changes to the fraction of ligand bound (f_b).

$$f_b = \frac{F_{obsd} - F_0}{F_\infty - F_0} \approx \frac{F_{obsd}}{F_\infty}$$

With ANS and bis(ANS), the fluorescence of the unbound ligand (F_0) is negligible. The values of $\bar{q}$ in the plots correspond to the observed fluorescence intensities of the equilibrium mixtures of free and bound bis(ANS) (same as F_{obsd}); q_0 is a calculated value for the fluorescence of an equivalent solution in which all the bis(ANS) is bound (same as F_∞). It is not

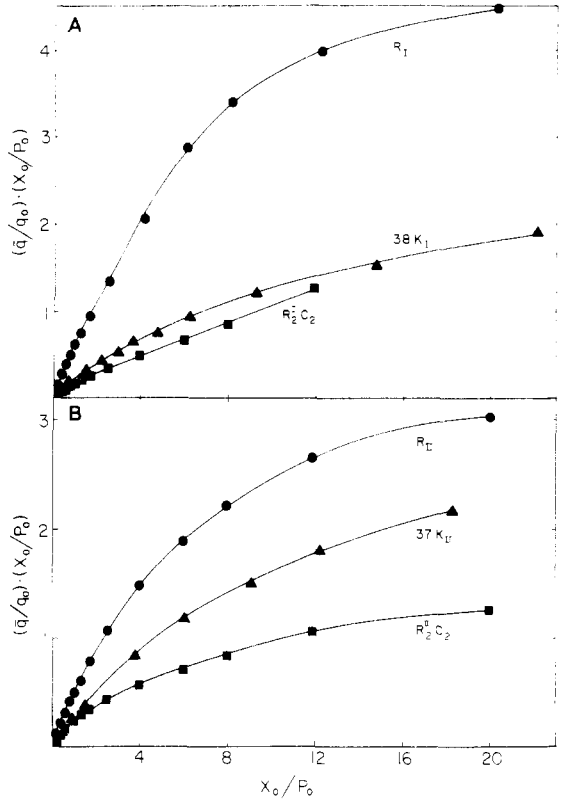

FIGURE 1: Bis(ANS) binding to (A) R^I (●), $R^I_2C_2$ (■), and $38K^I$ (▲) and to (B) R^{II} (●), $R^{II}_2C_2$ (■), and $37K^{II}$ (▲). The product $(\bar{q}/q_0)(X_0/P_0)$ is the number of moles of bis(ANS) bound per R^I monomer calculated from the fluorescence intensities (see text) and is directly proportional to the intensity corrected for the inner filter effect. X_0 is the total bis(ANS) concentration, and P_0 is the total protein concentration (fixed at 1.0 μM) in terms of regulatory subunit monomer. Conditions were 5 mM Mops, 0.1 N KCl, 0.5 mM EDTA, and 15 mM β-mercaptoethanol, pH 7, 25 °C. Excitation, 390 nm; emission, 480 nm.

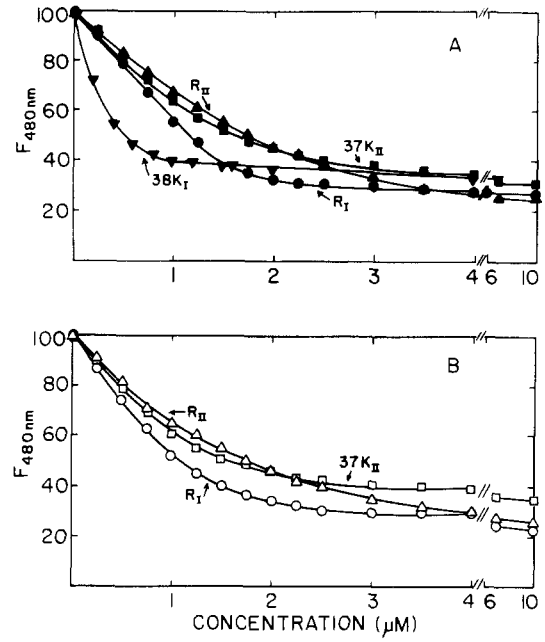

FIGURE 2: Titration of the bis(ANS) adsorbates of R^I, R^{II}, and the fragments with cAMP [(A), closed symbols] and ϵ-cAMP [(B), closed symbols]. Conditions were 10 μM bis(ANS) and 1 μM protein except for $38K^I$, which was 0.27 μM. R^I (●, ○), R^{II} (▲, △), $38K^I$ (▼), and $37K^{II}$ (■, □). See Figure 1 for other information.

feasible to measure F_∞ at varying degrees of saturation of the proteins. Varying concentrations of each protein were added to solutions containing 1 μM bis(ANS). The fluorescence of totally bound 1 μM bis(ANS), F_∞, was obtained by extrapolation to infinite protein concentration, using plots of F_{obsd}^{-1} vs. P_0^{-1}. With this approach, the degree of saturation of the protein binding sites is low. If the sites are heterogeneous, their relative frequencies of occupation are inversely proportional to the corresponding dissociation constants: high-affinity sites will contribute more to the value of F_∞ than low-affinity sites. Values of q_0 at other bis(ANS) concentrations are calculated by relating the value of F_∞ measured at 1 μM bis(ANS) to the fluorescence of the standards [either quinine sulfate or bis(ANS)–bovine serum albumin adsorbates]. In other words, the quantum yield of bis(ANS) is assumed to be independent of the number of (dye) molecules bound. When this is true, the product $(\bar{q}/q_0)(X_0/P_0)$ equals $\bar{n}$, the number of moles of ligand bound. Since high-affinity sites generally have higher quantum yields than low-affinity sites, the values of $\bar{n}_{app}$ represent *minimum* values, that is, $\bar{n}_{actual} \geq \bar{n}_{app}$.

The values of $\bar{n}_{app}$ in Figure 1 show that relatively large numbers of bis(ANS) molecules bind to each regulatory subunit. Scatchard plots extrapolate to 3.7 and 5.4 mol of bis(ANS) for R^{II} and R^I, respectively. Binding heterogeneity is quite likely where such large numbers of ligand molecules bind. We repeated the titration of R^{II} by using 1.2 mg of protein/mL in order to approximate the conditions for total binding. The fluorescence intensities at low values of X_0/P_0 were close to those calculated. At higher ratios, the intensities tended to be lower than expected with 20% deviation obtained when $X_0/P_0 = 4$ (data not shown). Since this experiment indicated a moderate dependence of quantum yield on the number of ligands bound, direct binding measurements were also performed by using the analytical ultracentrifuge equipped with a UV scanner. Comparisons of binding at the highest bis(ANS) concentration used (19 μM) showed that $\bar{n}$ obtained from sedimentation was higher than the value of $\bar{n}_{app}$ calculated from fluorescence (5.2 vs. 3.1). Sedimentation measurements carried out at even higher bis(ANS) concentrations, up to 100 μM, indicated that additional binding occurs, with 8–9 mol of bis(ANS) bound per mol of R^{II}.

In view of the above information, precise dissociation constants and stoichiometry cannot be obtained; however, average values of K_D for R^I and R^{II} are in the range of 10^{-6}–10^{-5} M. Figure 1 shows that measurements of bis(ANS) binding would be useful in studying the reconstitution of the holoenzyme. The binding of bis(ANS) by the proteolytic fragments $37K^{II}$ and $38K^{II}$ indicates that some of the bis(ANS) binding sites are either modified or lost as a result of the limited proteolysis.

Effect of Cyclic Nucleotides on Bis(ANS) Binding. Titration of the bis(ANS) complexes of R^I and R^{II} with cAMP (Figure 2A) and with 1,N^6-ethenoadenosine cyclic 3′,5′-phosphate (ϵ-cAMP) (Figure 2B) results in a 70–80% decrease in bis(ANS) fluorescence. The titrations of R^I are nearly linear, giving a stoichiometry of 1.6 mol of cAMP. Similar titrations using cGMP are illustrated in Figure 3. The addition of cGMP to R^I causes a sharp decline in bis(ANS) fluorescence to 30% of its initial value. The fluorescence changes resulting from the addition of cGMP to R^{II} are biphasic: there is an abrupt decline in fluorescence, to about 75% of the initial value at 10 μM cGMP, followed by a gradual decrease such that more than 40% of the initial fluorescence remains even at 400 μM cGMP. The latter part of this titration corresponds to $K \approx 150$ μM and extrapolates to the same total fluorescence change seen with cAMP.

Such fluorescence changes can result either from a decrease in quantum yield or from a dissociation of bis(ANS). Mea-

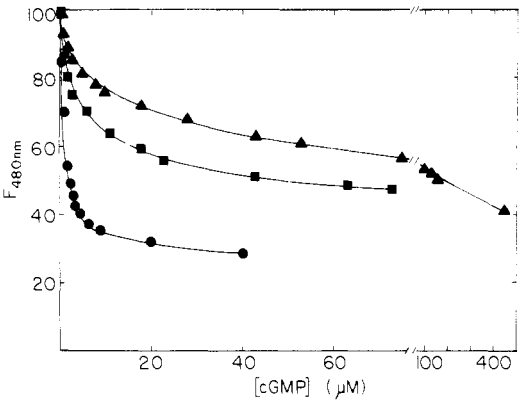

FIGURE 3: Titration of the bis(ANS) adsorbates with cGMP. Conditions were 10 μM bis(ANS) plus 1.0 μM R^I (●), R^{II} (▲), or $37K^{II}$ (■). Other information is given under Figure 1.

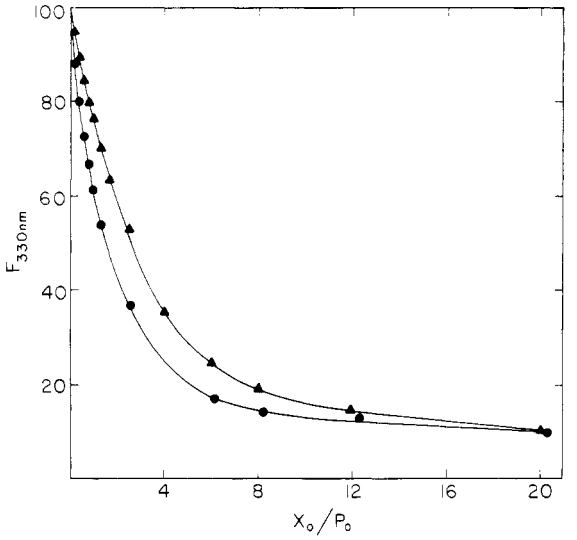

FIGURE 4: Quenching of the intrinsic protein fluorescence by bis(ANS). 1.0 μM R^I (●) or R^{II} (▲) was titrated with bis(ANS). The fluorescence intensities were corrected for inner filter effects, using parallel titrations of tryptophan standards with bis(ANS). Excitation, 290 nm; emission, 330 nm. See Figure 1 for other details.

surements of the intrinsic protein fluorescence can distinguish between these possibilities. The emission spectra of R^I and R^{II}, with maxima near 326 nm, are equally overlapped by two absorption bands of bis(ANS) and, to a lesser extent, by a third absorption band at 400 nm. This overlap shows that energy transfer from the tryptophan residues to bound bis(ANS) is favorable. Because large errors in estimation of the overlaps by the three absorption bands were evident, we did not calculate the characteristic energy transfer distance. However, it should be at least as large as the distance for ANS and tryptophan, i.e., 29 Å (Weber & Daniel, 1966). Figure 4 shows the decrease in intrinsic protein fluorescence (corrected for inner filter effects) accompanying the addition of bis(ANS) to both R^I and R^{II}. Comparison of these titrations with those in Figure 1 indicates that quenching is nearly complete before the subunits are saturated with bis(ANS). This nonlinear quenching means that each tryptophan donor can transfer energy to more than one bis(ANS) acceptor—a common occurrence whenever proteins bind more than one acceptor (Weber & Daniel, 1966). Since the absorption spectrum of bis(ANS) is comparatively insensitive to environmental changes, factors influencing the quantum yield of bis(ANS) should have slight effect on the efficiency of energy transfer. *Dissociation* of bis(ANS), in contrast, reduces the transfer

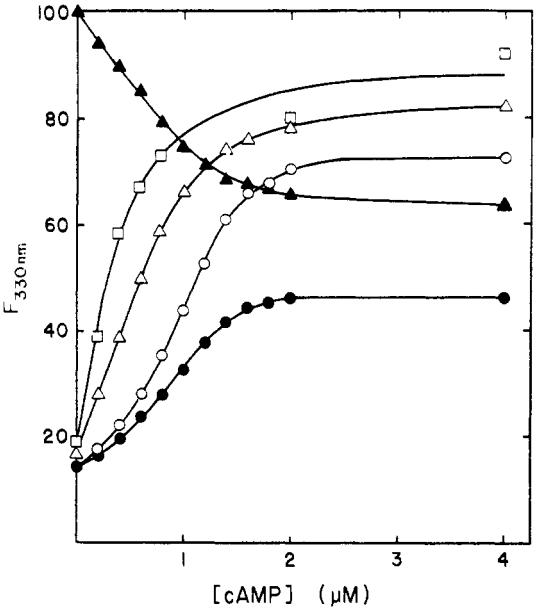

FIGURE 5: Recovery of the intrinsic protein fluorescence on addition of cAMP to the bis(ANS) adsorbates. The *observed* intensities obtained with 1.0 μM R^I (●), R^{II} (△), and $37K^{II}$ (□) are shown. cAMP by itself quenches the fluorescence of R^I [see (▲)] but not R^{II} (LaPorte et al., 1980). The open circles (○) describe the recovery expected for R^I if quenching by cAMP had not occurred. The bis(ANS) concentration was fixed at 10 μM. Excitation, 290 nm; emission, 330 nm. See Figure 1 for other conditions.

efficiency to zero. The recovery of intrinsic protein fluorescence occurring when the bis(ANS) adsorbates of R^I, R^{II}, and $37K^{II}$ are titrated with cAMP is illustrated in Figure 5. R^{II} recovers ~80% of its initial fluorescence and R^I more than 70% when quenching by cAMP is considered. These extensive recoveries indicate that most of the bound bis(ANS) is released upon binding of cAMP. Comparison with the quenching curves in Figure 4 suggests that less than 1 mol of bis(ANS) remains bound in each case. An alternate explanation, i.e., that a conformational change increases the distance between donor and acceptor, is unlikely since several bis(ANS) molecules would have to be displaced a considerable distance from the tryptophan donor.

Random displacement of all the bis(ANS) molecules by cAMP should reverse the fluorescence changes seen in the original titrations of R^I and R^{II}. Comparison of Figures 4 and 5 indicates that the displacement is not random and that the fluorescence intensity is recovered more rapidly than expected with both R^I and R^{II}. The titrations shown in Figure 5 reflect the characteristic cAMP binding properties of R^I and R^{II}. R^{II} displays noninteracting high- and low-affinity sites for cAMP while R^I binds the nucleotide cooperatively [cf. reviews by Hoppe & Wagner (1979) and Smith et al. (1981)]. The addition of the first mole of cAMP to R^{II} accounts for 80% of the total fluorescence recovery, suggesting either that most of the bis(ANS) is displaced after occupation of the high-affinity site by cAMP or that the remaining bis(ANS) is located further away from tryptophan. The symmetric sigmoidal titration of R^I is consistent with cooperative binding and the resulting distribution of the population into *two* major fluorescent species—$R^I_2(cAMP)_4$ and free R^I—which are either fully saturated or unsaturated (Weber & Daniel, 1966; G. Weber, unpublished calculations). The relative proportions of the two components would vary directly with the degree of saturation, explaining the nearly linear rate of recovery through the mid-saturation range of ~20–80%. The deviations from linearity seen at the extremes of the titration curve are con-

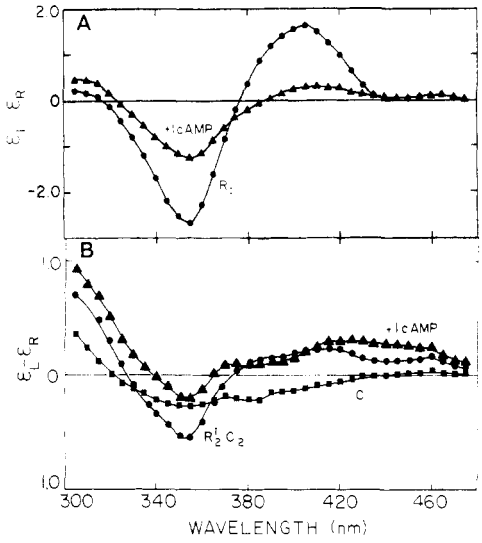

FIGURE 6: Circular dichroism spectra of solutions containing (A) 12 μM R^I, 60 μM bis(ANS), and no additions (●) or 12 μM cAMP (▲) and (B) 6 μM $R^I{}_2C_2$, 60 μM bis(ANS), and no additions (●) or 6 μM cAMP (▲). $\epsilon_L - \epsilon_R$ in cm^{-1} M^{-1} was calculated by using the total bis(ANS) concentration. The spectrum obtained with 12 μM catalytic subunit (■) is also shown. See Figure 1 for other conditions.

sistent with observations and predictions on the disappearance of cooperativity at the limits of the saturation range [cf. review by Wyman (1964)].

Effect of Magnesium on Bis(ANS) Binding. The addition of magnesium enhances the fluorescence of bis(ANS) bound to R^I, R^{II}, and $37K^{II}$ by 40–50%. The hyperbolic saturation curves for R^{II} and $37K^{II}$ are superimposable; apparent dissociation constants for magnesium, calculated from linear double-reciprocal plots of the fluorescence changes vs. magnesium concentration, are 12 and 11 mM for R^I and R^{II}, respectively. Addition of cAMP to these mixtures results in the slow release of most of the bound bis(ANS) over a 10-min period. The fluorescence intensity at equilibrium is the same as that attained in the earlier experiments (see Figure 2), where equilibrium was reached within a few seconds after addition of cAMP.

Circular Dichroism Spectra of Bis(ANS) Complexes. The free bis(ANS) molecule is symmetric and has no optically active absorption bands. Binding, however, induces asymmetry. The accurate differential measurements obtained in circular dichroism (CD) are useful in the resolution of ligand binding to multiple sites. We have previously used CD to demonstrate heterogeneous binding of ANS by bovine serum albumin. (Anderson, 1969) and to resolve the binding of bis(ANS) to the two subunit types in the hybrids of lactate dehydrogenase (Anderson, 1971). The success of these previous applications indicated that CD measurements would be useful in experiments dealing with the binding of both cAMP and the catalytic subunit by the regulatory subunits and their proteolytic fragments. Figures 6A and 7A show the distinctive CD spectra of the complexes of bis(ANS) with R^I and R^{II}. The addition of cAMP causes a decrease in the absolute values of $\epsilon_L - \epsilon_R$. The change in the 358-nm CD band of the R^{II}–bis(ANS) adsorbate is nearly proportional to the concentration of added cAMP, while corresponding changes in the 380–460-nm region are clearly nonlinear. The holoenzymes have characteristic spectra (Figures 6B and 7B) with absolute values of $\epsilon_L - \epsilon_R$ 5–10-fold smaller than those obtained with R^I and R^{II}. This difference suggests that most of the bis(ANS) molecules responsible for the CD of R^I and R^{II} dissociate when the holoenzymes are formed.

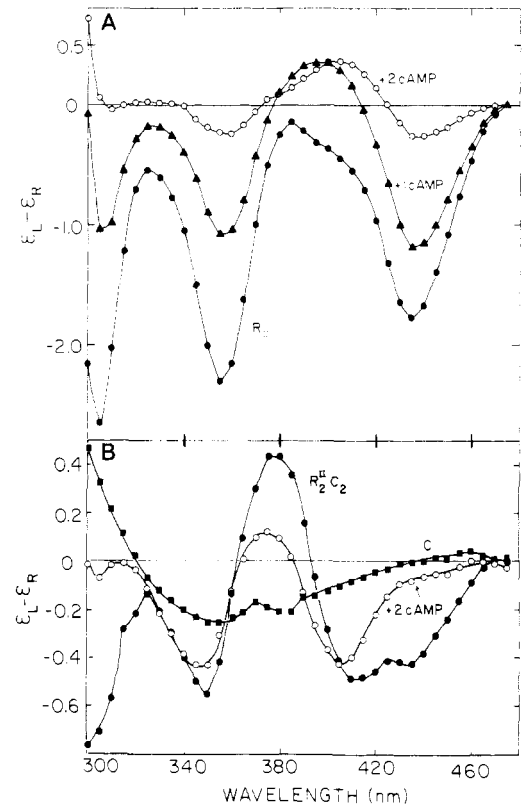

FIGURE 7: Circular dichroism spectra of solutions containing (A) 12 μM R^{II}, 60 μM bis(ANS), and no additions (●), 12 μM cAMP (▲), or 24 μM cAMP (○) and (B) 6 μM $R^{II}{}_2C_2$, 60 μM bis(ANS), and no additions (●) or 24 μM cAMP (○). The spectrum of a 12 μM solution of the catalytic subunit (■) is also shown. See Figure 1 for other conditions.

The CD spectrum of the adsorbate of bis(ANS) with the 37-kdalton fragment of R^{II} (Figure 8A) is almost identical with that obtained with native R^{II}, except between 380 and 410 nm. The effects of cAMP on the CD of $37K^{II}$ are nearly identical with those seen with R^{II}, indicating that the fragment binds most of the optically active bis(ANS) molecules also bound by R^{II}.

The addition of an equivalent amount of catalytic subunit to $37K^{II}$ results in a CD spectrum different from that predicted by summation of the separate spectra of C and $37K^{II}$ (Figure 8B). We calculated the amount of $37K^{II}$ bound to C by assuming that the $37K^{II}$–C complex gives the same CD spectrum with bis(ANS) as that obtained with $R^{II}{}_2C_2$. The average CD of a mixture of components can be calculated from their mole fractions and individual values of $\epsilon_I - \epsilon_R$, i.e., $\epsilon_L - \epsilon_R = f_C(\epsilon_L - \epsilon_R)_C + f_{37K^{II}}(\epsilon_L - \epsilon_R)_{37K^{II}} + f_{K^{II}C}(\epsilon_L - \epsilon_R)_{K^{II}C}$. In this instance, $f_C = f_{37K^{II}}$ and $f_{K^{II}C} = 1 - f_{37K^{II}}$. We used the 358-nm CD band for these calculations since it is preserved in $37K^{II}$ and gives a large difference between the signals produced by the $R^{II}{}_2C_2$ complex and the separate subunits, i.e., $-1.5 = (1 - f_{K^{II}C})(-0.24 - 2.1) + f_{K^{II}C}(-0.2)$. Solution of this equation indicates that the fraction of the fragment bound by $37K^{II}$ is 0.39.

Sedimentation Studies. Sedimentation velocity experiments were carried out to compare the effectiveness of cAMP and bis(ANS) in promoting the dissociation of $R^{II}{}_2C_2$. The values of $s_{20,w}$ in Table II show that bis(ANS) does not dissociate the holoenzyme; on the contrary, a 13% increase in $s_{20,w}$ indicates that some association occurs. By contrast, addition of cAMP to the mixture results in considerable dissociation. Complete dissociation would require a large excess of cAMP even in the absence of bis(ANS).

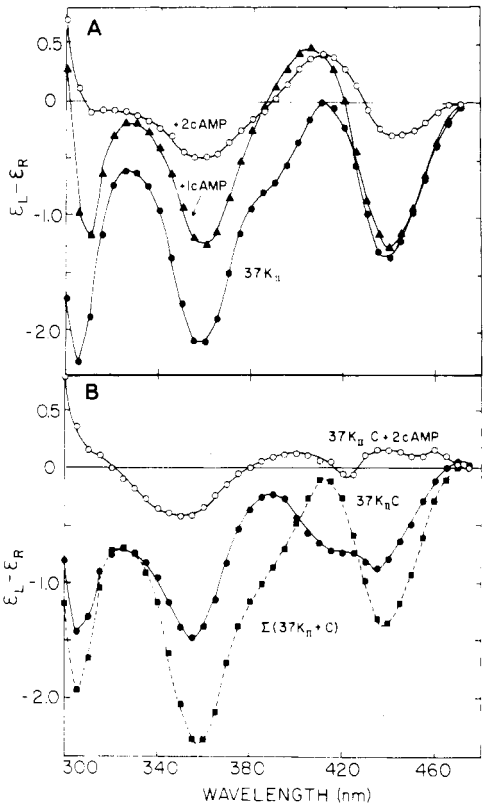

FIGURE 8: Circular dichroism spectra of solutions containing (A) 12 μM 37K^{II}, 60 μM bis(ANS), and no additions (●), 12 μM cAMP (▲), or 24 μM cAMP (○) and (B) 12 μM 37K^{II}, 12 μM C, 60 μM bis(ANS), and no additions (●) or 24 μM cAMP (○). The dotted line represents the addition of the individual spectra of bis(ANS) with 37K^{II} alone and with C alone. See Figure 1 for other conditions.

Table II: Sedimentation Coefficients of $R^{II}{}_2C_2$ in the Absence and Presence of Bis(ANS) and cAMP[a]

protein	[bis(ANS)] (μM)	[cAMP] (μM)	$s_{20,w}$ (S)
$R^{II}{}_2C_2$			6.69 ± 0.13
$R^{II}{}_2C_2$	100		7.55 ± 0.14
$R^{II}{}_2C_2$	100	40	5.86 ± 0.19
$R^{II}{}_2C_2$		476	5.00 ± 0.05
$R^{II}{}_2$			4.19 ± 0.02
$R^{II}{}_2$		18	3.91 ± 0.02
C			3.37 ± 0.02

[a] Conditions were 5 mM Mops, 0.1 N KCl, 50 mM β-mercaptoethanol, pH 7 (25 °C), and 2 mg/mL protein. Rotor speed, 52 000 or 60 000 rpm.

Discussion

The binding of bis(ANS) by both types of regulatory subunit of cAMP-dependent protein kinase results in large increases in the fluorescence quantum yield and the optical activity of the ligand. Interaction of the regulatory subunits with either cAMP or the catalytic subunit causes most of the fluorescence enhancement and optical activity to disappear. Actual dissociation of bis(ANS), accompanying the binding of cAMP or the catalytic subunit, is the simplest explanation of these changes. However, the stoichiometry of bis(ANS) binding, a minimum of four to six molecules of bis(ANS) per monomer, is larger than the stoichiometries of binding for the other two ligands [cf. Glass & Krebs (1980)]. Another explanation could be that cAMP and the catalytic subunit induce conformational changes in R affecting the quantum yield and optical activity of the adsorbed bis(ANS). However, cAMP appears to remove a significant portion of the bis(ANS), as indicated by the recovery of 70–80% of the intrinsic protein fluorescence. This recovery of protein fluorescence shows that an appreciable amount of bis(ANS) dissociates when cAMP binds. Both the residual quenching and fluorescence enhancement of bis(ANS) show that some of the dye remains bound even at saturating cAMP concentrations; the data in Figure 4 suggest that less than one molecule per monomer remains after additions of cAMP. If true, the number of moles of bis(ANS) displaced would exceed the amount of cAMP bound. However, quantitative interpretation of the residual quenching is difficult since the displacement of bis(ANS) by cAMP is nonrandom. The fraction of bis(ANS) that remains bound could exceed the amount estimated from the quenching if the residual bis(ANS) molecules were less effective in energy transfer. For instance, they could be more distant from the tryptophan residues than those molecules that had been released. In lactate dehydrogenase, 12 bis(ANS) molecules are bound per tetramer with about equal affinities. In the isozyme M_4, *four* of these account for most of the observed fluorescence and CD; they are involved in the reversible polymerization of the enzyme and are displaced by NADH (Anderson, 1971; Lu & Anderson, 1973). A similar situation of heterogeneous bis(ANS) binding may exist with the regulatory subunits of protein kinase.

Sedimentation velocity experiments showed that bis(ANS) is ineffective in dissociating type II protein kinase; instead, a slight association may occur. However, significant dissociation occurs when cAMP is subsequently added.

Bis(ANS) is not an obvious analogue of either cAMP or NADH. Although binding to nucleotide binding sites can account for most of the fluorescence enhancement and optical activity in both lactate dehydrogenase and the regulatory subunits of protein kinase, attachment to other sites, as well as altered interactions with the nucleotide site, may occur. Inhibition of several dehydrogenases and kinases by Cibacron Blue and related compounds suggested that only a part of the Cibacron Blue molecule is involved in specific interactions with the nucleotide binding sites while other parts bind nonspecifically (Beissner & Rudolph, 1978). The binding of hemin by cAMP-dependent protein kinase (Datta et al., 1977) may similarly involve both specific and nonspecific interactions. Bornmann & Hess (1977) suggested that dyes such as Cibacron Blue mimic the adenosine moiety of NAD^+, ADP, and ATP rather than the entire dinucleotide. X-ray diffraction studies showed that ANS, 5-iodosalicylic acid, and tetraiodofluorescein occupy the adenine subsite in both alcohol and lactate dehydrogenases (Wasserman & Lentz, 1971; Einarsson et al., 1974).

The major difference between cAMP and cGMP binding lies in the concentrations required to displace bis(ANS). R^{II} has high- and low-affinity cAMP binding sites which are spectroscopically resolvable (LaPorte et al., 1980; Smith et al., 1981; Bohnert et al., 1982). The efficient displacement of a portion of the bis(ANS) by low concentrations of cGMP suggests that the high-affinity site of R^{II} also strongly binds cGMP. In contrast, the excess of cGMP needed to bring about maximum release of bis(ANS) indicates that the low-affinity site binds cGMP poorly, with an apparent dissociation constant on the order of 150 μM.

Since binding of cAMP to both low- and high-affinity sites is necessary for the dissociation of $R^{II}{}_2C_2$ (Smith et al., 1981), the above data suggest why cGMP is inefficient in activating the type II enzyme. Nonidentity of the cAMP and cGMP sites is unlikely since the total fluorescence changes are the same for the two nucleotides. The adenine and guanine rings may

interact differently with the same binding site. London & Schmidt (1972) proposed a model for aspartate transcarbamylase in which the N-1 of adenine interacts with an electrophilic group on the enzyme to stabilize the anti conformation of ATP while the N-7 of guanine interacts with the same group to stabilize the syn conformation of GTP. No information is available on the conformation of the cyclic nucleotides when bound to the protein kinases.

The positive cooperativity of cAMP binding to R^I described here and by others (Hoppe & Wagner, 1979) and the finding that cGMP binds overall more effectively to R^I than to R^{II} suggest that cGMP-dependent protein kinase might be more closely related to $R^I{}_2C_2$ than to $R^{II}{}_2C_2$. Limited proteolysis studies as well as phosphorylation studies have indicated that the structural organization of R^I is similar to that of the regulatory domain of cGMP-dependent protein kinase (Potter & Taylor, 1980; Geahlen et al., 1981; Gill et al., 1981). The binding of cGMP to the cGMP-dependent protein kinase is also cooperative (McCune & Gill, 1979; Gill et al., 1981); there is an additional ATP site on the enzyme although it is not of high affinity like the one found in the type I kinase (Gill et al., 1981).

The fluorescence intensities of the adsorbates of R^I, R^{II}, and $37K^{II}$ increase in the presence of Mg^{2+}. This probably reflects increased binding of bis(ANS) rather than quantum yield changes since the initial quantum yields of the complexes are high. The formation of additional binding sites is also unlikely since the addition of cAMP decreases the fluorescence to the same value obtained in the absence of Mg^{2+}. The time-dependent displacement of bis(ANS) by cAMP in the presence of magnesium suggests that slow conformational changes are taking place. This is the first evidence for Mg^{2+} binding by the isolated regulatory subunits; whether the effect is specific for Mg^{2+} is not known. The possibility that other divalent cations such as Ca^{2+} might bind is currently being explored.

The 37-kdalton fragment of R^{II} is similar to intact R^{II} in its interaction with bis(ANS) and cAMP. Although fluorescence measurements showed that less bis(ANS) may be bound to $37K^{II}$, CD spectra indicated that most of the optical activity characteristic of the R^{II} adsorbate is preserved in $37K^{II}$. CD measurements indicated that 39% of the fragment binds to C, compared to 32% estimated from sedimentation experiments (Bohnert et al., 1982). Changes in bis(ANS) fluorescence and fluorescence polarization with ϵ-cAMP (Bohnert et al., 1982) indicated that the extent of fragment C interaction is 24 and 15%, respectively. All measurements indicate heterogeneity of the fragment, with varying degrees of binding competence. Variations in the values obtained may also reflect the age of the preparation used. Heterogeneous cleavage near the hinge region, where proteolysis presumably occurs, could have a dramatic effect on the binding of the catalytic subunit (Taylor et al., 1981; Bohnert et al., 1982).

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